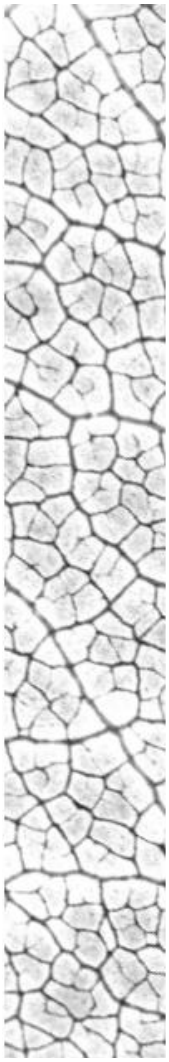

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Isolation and characterization of *Staphylococcus haemolyticus* from urinary tract infection

Mohammed Twfeek Abid Alhusain [1], Firas Nabeeh Jaafar [1], Afrah Abdulridha Ajeel Hassan [1*]

[1] Biology Department, College of Science, Mustansiriyah University, Baghadad, IRAQ
*Corresponding author: afrahalmaliki79@uomustansiriyah.edu.iq

Abstract
Staphylococcus haemolyticus is a member of coagulase negative staphylococci colonize skin. The present study aimed to isolate and characterize *S. haemolyticus* from urinary tract infection (UTI). One hundred forty clinical samples isolated from UTI were included in this study. The colony morphology, gram stain and coagulase enzyme were tested against the typical colonies. All the selected isolated were subjected to Vitec 2 compact for identification and antibiotics sensitivity testing. The formation of biofilm was tested for all the isolates of *S. haemolyticus*. Biofilm formulation has been examined on S. haemolyticus isolates. The effect of different concentrations of carbohydrates, pH and NaCl on formation of biofilm was further analyzed. Among the collected samples, thirty were identified as *S. haemolyticus*. All tested strains of *S. haemolyticus* were resistant to antibiotics except two tigecycline and linezolid. All the isolates produced biofilm, one isolate was the highest with OD value 0.7. The biofilm formation was affected by the physio-chemical factors. *S. haemolyticus* isolated from UTI subjects. Multi resistant antibiotics were observed. Further studies are necessary to identify the anti-biofilm.

Keywords: biofilm, antibiotic sensitivity testing, multidrug resistance, Vitec 2 compact

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INTRODUCTION

The urinary tract is the furthermost usual location of bacterial disease in human beings (Dehghanrad et al., 2019; Wagenlehner, 2016). Urinary tract infections (UTIs) are one of the foremost predominant infections, affecting a large number of females (Horcajada et al. 2013; Foxman, 2010). Forty to fifty percent of the female are predicated to be diseased with UTI throughout their lifetime (Griebling, 2005). During 2007, around 8 million patients (84 % female) were approached physician. The occurrence of lower UTI starts when bacteria colonize the periurethral area due to stool contamination from the digestive tract, leads to the rise of microorganisms within the urethra and bladder, initiating cystitis. Untreated cystitis may develop to an upper UTI called pyelonephritis (Foxman, 2010; Andersen, 2019).

Staphylococcus haemolyticus is one of the common and continuous aetiological agents of staphylococcal infections. Moreover, *S. haemolyticus* considered as the most isolated and significant species of coagulase-negative staphylococci (CoNS). Among CoNS species, *S. haemolyticus are the highest isolated* nosocomial antibiotic resistant bacteria (Cavanagh et al. 2016; Cavanagh et al.2019). The vital virulence factors of *S. haemolyticus* appears to be absent compared with other species of staphylococci. Multi resistance is the core role in the estimation of a risk made by *S. haemolyticus*. Furthermore, the adaptation and capability of *S. haemolyticus* to endure in the hospital atmosphere and medical instruments made it the essential factor in nosocomial contagions (Barros et al. 2015; Czekaj et al. 2015, Ghosh, 2018).

UTIs are widely recognized bacterial contagions around the globe. Their treatment is getting to be more complicated as resistance for regular antimicrobials are expanding (Wagenlehner et al. 2016). The increment of antimicrobial resistances and multi-drug resistance pathogens in UTI is related to greater paces of deficient observational treatment because of disabled antimicrobial scope (Horcajada et al. 2013).

The expanding number of CoNS in medical clinics is firmly identified with their antimicrobial resistance and the capacity to persist in hospitals. Numerous researchers revealed *S. haemolyticus* strains as resistant to at least one antibiotics i.e. Tetracyclines, penicillins, cephalosporins, macrolides, fosfomycin, quinolones, glycopeptides and aminoglycosides (Cavanagh et al. 2016; Seng et al. 2017). Strains of *S.*

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Table 1. Range of different concentration of used antimicrobial agents

Antibiotics	Concentration range (µg/ml)
1. Benzylpencillin	0.125-1
2. Oxacillin	0.5-2
3. Levofloxacin	0.25-8
4. Linezolid	0.5-2
5. Tigecycline	0.25-1
6. Moxifloxacn	0.25-8
7. Teicoplanin	1-16
8. Nitrofurantoin	16-64
9. Gentamicen	8-64
10. Erytromycn	0.25-2
11. Vancomycin	1-16
12. Fusidic acid	0.5-4
13. Tobramycin	8-64
14. Clindamycin	0.25-0.5
15. Tetracyclin	1-16
16. Rifampicin	0.25-2
17. Trimethoprim- sulfmethozaole	16/404-32/608

haemolyticus were reported to be resistant to most of commercial antibiotics (Cavanagh et al. 2019; Maleki et al. 2019). In respect to the above, the aim of the present study was to isolate and characterize *Staphylococcus haemolyticus* from subjects complaining urinary tract infection.

MATERIALS AND METHODS

Sample Collection and Screening

One hundred forty urine samples (60 % female and 40 % male) aged range between 20 to 70 years old were collected from patients suffering UTI from laboratories of Al-Nokhba diagnostics center, Baghdad, Iraq, between March 2018 to May 2019. All the subjects were fully aware and educated about the current study and formal consent form were signed. The urine samples were cultured on MacConkey, Blood agar and Mannitol salt agar (HiMedia, India). The colony morphology, shape, size, swarming phenomenon and texture of the bacteria were observed. Single colonies were picked, stained with gram stain and examined under a light microscope then tested with coagulase enzyme.

Vitek 2 Compact

All the screened isolates were subjected for identification and antibiotics sensitivity testing by VITEK 2 compact system (bioMérieux, France) according to the instruction provided by the manufacturer. Three to five fresh colonies from each isolate were transferred into two tubes containing 3 ml normal saline. The turbidity has been set by DensiCHEK Plus to 0.5 as per McFarland standard. The suspension was inoculated into the Vitek 2 compact with gram positive identification test and sensitivity test (AST-P640) cassettes (bioMérieux, France). Interpretation of results was performed according to VITEK 2 compact system special software as explained by the manufacturer's instructions. The concentration ranges of the tested antimicrobial agents are shown in **Table 1**.

Biofilm Formation Assay

The formation of biofilm of *S. haemolyticus* was assessed by 96-well plate as described by O'Toole, (2011) with few modifications. Briefly, isolates from the overnight plate were inoculated into 10 ml of Loria Bertani (LB) broth (HiMedia, India) and incubate 37 Cº for 24 h. The culture broth was adjusted to 0.01 O.D and then inoculated into LB broth and incubated in moist chamber at 37 Cº for 24 h. After incubation, the suspension was carefully removed with a multichannel pipette. Distilled water was used to wash the wells and then allowed to stain with 0.1 % crystal violet. The wells were washed with distilled water and then dried at room temperature. The stained biofilm was then quantified by dissolving it with 250 µl of 95 % ethanol and measure the OD at 570 nm. The bacteria with OD value <0.120, 0.120-0.240 and 0.240 are non-biofilm, moderate biofilm and strong biofilm producer, respectively.

Effect of Carbohydrates, NaCl and pH on Biofilm Formation

The formation of biofilm was observed by adding, different concentrations of carbohydrates, NaCl and pH as a supplement in the media containing biofilm producing bacteria. The method was followed as explained by Sonkusale and Tale, (2015). Carbohydrates such as lactose, maltose, sucrose, glucose, fructose, galactose and starch with different concentrations (1 %, 2 %, 3 % and 4 %). Different concentrations of NaCl are 0.5, 1, 1.5, 2, 2.5, 3 and 3.5. The concentration of pH involved in this study are 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5 and 8. The OD of all the concentrations was measured at 570 nm.

RESULTS

Out of 140 samples, 31 isolates (43.3 % females and 56.6 % males) were identified as *S. haemolyticus*. The isolates were coagulase negative gram positive cocci. The sensitivity testing of 17 antibiotics was listed in **Table 2**. All the isolates produced moderate to strong biofilm, the average of biofilm is 0.42. Outcome of various levels of carbohydrates, NaCl and pH on biofilm were illustrated in **Tables 3**, **4** and **5**, respectively.

DISCUSSION

S. haemolyticus are bacterial normal flora inhabit the skin and considered as opportunistic microorganisms (Cavanagh et al. 2019). In the present study, thirty isolates (43.3 % females and 56.6 % males) of coagulase negative staphylococci (*S. haemolyticus*) isolated from UTI patients were detected (**Table 2**). The presence of *S. haemolyticus* in males was higher than females that is maybe due to the presence of other species CoNS in female. However, *S. haemolyticus* was previously isolated from a male with a higher rate than female (Leighton and Little, 1986; Pindar and Viau,

Table 2. Antibiotics sensitivity testing of *S. haemolyticus*

Tested Antibiotics	Resistant (No. of strains)	Sensitive (No. of strains)	Intermediate (No. of strains)
Benzylpencillin	31	0	0
Oxacillin	31	0	0
Gentamicen	15	08	08
Tobramycin	15	13	03
Levofloxacin	22	02	07
Moxifloxacn	19	10	02
Erytromycn	30	0	01
Clindamycin	23	07	01
Linezolid	0	31	0
Teicoplanin	02	24	05
Vancomycin	02	25	04
Tetracyclin	28	03	0
Tigecycline	31	0	0
Nitrofurantoin	01	28	02
Fusidic acid	30	01	0
Rifampicin	07	22	02
Trimethoprim-Sulfmethozaole	17	14	0

Table 3. Effect of various concentrations of carbohydrates on biofilm

Carbohydrates	1%	2%	3%	4%
Lactose (OD value)	0.547	0.506	1.419	0.951
Maltose (OD value)	0.998	0.8895	0.908	1.0625
Sucrose (OD value)	0.531	0.721	0.571	1.149
Glucose (OD value)	0.714	0.502	0.5255	1.333
Fructose (OD value)	1.149	0.875	0.8505	1.3185
Galactose (OD value)	0.7755	0.718	0.8715	0.84
Starch (OD value)	0.8965	0.5	1.062	0.727

2018). Furthermore, *S. haemolyticus* is well-known to colonize urethra and periurethra in females and males which can lead to genitourinary infections in both the genders (Becker et al. 2014). In the current study, the identification of the isolates was by Vitec 2 compact, which exhibited a high accuracy of species identification (Bazzi et al. 2017; Monteiro et al. 2016).

The antibiotics susceptibility testing were performed by Vitec 2 (**Table 2**). Almost all the *S. haemolyticus* isolates exhibited multi-resistant profiles (28/30; 93.3%). Among the tested antibiotics, two (Benzylpenicillin and oxacillin) showed resistant against all the isolates (100 %) and other two antibiotics (fusidic acid, erythromycin) displayed resistance to 96.6% of the isolates. According to a review paper conducted by Czekaj et al. (2015), estimated the resistant of different antibiotics against *S. haemolyticus* during 1989-2010. The study revealed that penicillin (87.6-95 %), Erythromycin (79-85.7 %), fusidic acid (31-50 %), Gentamicin (79-92.9 %) developed increment in antibiotics resistant level. Which shows similar results with the current study (**Table 2**). While tetracycline (75-28.6 %) and trimethoprim (63-57.1 %) were showing decrement level of antibiotics resistance with the years which is not in accordance with our present study. Maleki et al. (2019) reported erythromycin as a resistant antibiotic against isolates of *S. haemolyticus* (Maleki et al. 2019), which is in agreement to our study. Bischoff et al. (2018) found 36.5 % of 137 isolates of UTI multidrug resistance.

Table 4. Effect of different concentration of NaCl on biofilm

NaCl concentration	OD value
0.5	0.8845
1	0.8985
1.5	1.4285
2	0.785
2.5	1.106
3	0.7585
3.5	0.7355

Table 5. Effect of different pH values on biofilm formation

pH concentration	OD value
4	0.673
5	1.025
5.5	0.994
6	0.71
6.6	0.862
7.5	0.9085
8	0.636

In another hand, all the isolates of *S. haemolyticus* were sensitive to two antibiotics named tigecycline and linezolid (**Table 2**).

The present results revealed that all the isolates of *S. haemolyticus* were capable to yield biofilm, one of which displayed a high accumulation of biofilm (OD 0.7). However, previous reports demonstrated that 74 % and 67 % of the study population of *S. haemolyticus* isolates were forming a biofilm (Silva et al. 2013). In addition, some researchers attributed the antibiotics multi resistance isolates with their formation of biofilm (Silva et al. 2013; Paharik and Horswill, 2016).

In the current study, the higher level of biofilm production was found in sample no A225, which was selected as a model example. All the clinical strains of *S. haemolyticus* showed formation of biofilm (**Tables 3**, **4** and **5**). The biofilm showed high formation in the 3 % concentration of lactose, galactose and starch and was higher in the concentration of4 % for maltose, sucrose, fructose and glucose (**Table 3**). The study also revealed 1.5 NaCl and pH 5 were perfect concentrations to produce higher biofilm (**Tables 4** and **5**).

Results revealed that the physical factors like different concentrations of pH, NaCl and carbohydrates are important for producing biofilm which is in agreement with previous reports (Khangholi and Jamalli, 2016; Cai et al. 2016). Hence, infections caused by *S. haemolyticus* can be controlled by manipulating these factors.

CONCLUSION

The present data showed that *S. haemolyticus* isolates collected from subjects suffering from UTI, exhibited high-level multi-resistance antibiotics and capability to produce biofilms. The accumulative population of these multi-resistant isolates of *S. haemolyticus* may lead to addressing it as an opportunistic pathogen. Further researches are necessary to study the biofilm formation and their inhibition.

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